Claims

1. A method for constructing a cell comprising at least two copies of a gene of interest stably integrated into the chromosome in different positions, the method comprising the steps of:

a) providing a host cell comprising at least one chromosomal copy of the gene of interest, and comprising one or more conditionally essential chromosomal gene(s) which has been altered to render the gene(s) non-funtional;

b) providing a DNA construct comprising:

i) an altered non-functional copy of the conditionally essential gene(s) of step a); and

ii) at least one copy of the gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence located on the host cell chromosome adjacent to the gene(s) of step a); wherein a first recombination between the altered copy of i) and the altered chromomosomal gene(s) of step a) restores the conditionally essential chromosomal gene(s) to functionality and renders the cell selectable;

c) introducing the DNA construct into the host cell and cultivating the cell under selective conditions that require a functional conditionally essential gene(s); and

d) selecting a host cell that grows under the selective conditions of the previous step ; wherein the at least one copy of the gene of interest has integrated into the host cell chromosome adjacent to the gene(s) of step a); and optionally

e) repeating steps a) to d) at least once using a different chromosomal gene(s) in step a) in each repeat.

2. A method for constructing a cell comprising at least two copies of a gene of interest stably integrated into the chromosome in different positions, the method comprising the steps of:

a) providing a host cell comprising at least one chromosomal copy of the gene of interest;

b) altering a conditionally essential chromosomal gene(s) of the host cell whereby the gene becomes non-funtional;

c) making a DNA construct comprising:

i) an altered non-functional copy of the chromosomal gene(s) of step b); and

ii) at least one copy of the gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence adjacent to the

gene(s) of step b); wherein a first recombination between the altered copy of i) and the altered chromomosomal gene(s) of step b) restores the chromosomal gene(s) to functionality and renders the cell selectable;

d) introducing the DNA construct into the host cell and cultivating the cell under selective conditions that require a functional gene(s) of step b); and

e) selecting a host cell that grows under the selective conditions of step d); wherein the at least one copy of the gene of interest has integrated into the host cell chromosome adjacent to the gene(s) of step b); and optionally

f) repeating steps a) to e) at least once using a different chromosomal gene(s) in step b) in each repeat.

3. The method of claim 1 or 2, wherein subsequent to the step of introducing the DNA construct and cultivating the cell under selective conditions, or subsequent to the step of selecting a host cell, a second recombination takes place between the DNA fragment and the homologous host cell DNA sequence.

4. The method of claim 3, where the DNA construct further comprises at least one marker gene which is located in the construct such that it is recombined out of the chromosome by the second recombination.

5. The method of claim 4, wherein the at least one marker gene confers resistance to an antibiotic, preferably the antibiotic is chosen from the group consisting of chloramphenicol, kanamycin, ampicillin, erythromycin, spectinomycin and tetracycline.

6. The method of claim 4 or 5, wherein a host cell is selected which grows under the selective conditions, and which cell does not contain the at least one marker gene in the chromosome.

7. The method of any of claims 1 - 6, where the DNA construct further comprises at least one marker gene located between the altered copy and the DNA fragment, and wherein the at least one marker gene is flanked by nucleotide sequences that are recognized by a specific resolvase, preferably the nucleotide sequences are *res*.

8. The method of claim 7, wherein the at least one marker gene is excised from the chromosome by the action of a resolvase enzyme subsequent to selecting a host cell that grows under the selective conditions.

9. The method of any of claims 1 - 8, wherein the gene of interest originates from the host cell.

10. The method of any of claims 1 – 9, wherein the gene of interest encodes an enzyme, preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme, and more preferably an enzyme with an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase.

11. The method of any of claims 1 – 10, wherein the selected host cell that grows under the selective conditions comprises substantially no exogenous DNA, preferably less than 500 basepairs per integrated gene of interest, more preferably less than 300 bp, even more preferably less than 100 bp, still more preferably less than 50 bp, more preferably less than 25 bp per integrated gene of interest, or most preferably no exogenous DNA.

12. The method of any of claims 1 – 10, wherein the selected host cell that grows under the selective conditions comprises DNA only of endogenous origin.

13. The method of any of claims 1 – 12, wherein the conditionally essential chromosomal gene(s) of the host cell is altered by partially deleting the gene(s), or by introducing one or more mutations in the gene(s).

14. The method of any of claims 1 – 13, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered encodes a D-alanine racemase, preferably the gene(s) is

a *dal* homologue from a *Bacillus* cell, more preferably the gene is homologous to *dal* from *Bacillus subtilis,* and most preferably the gene(s) is the *dal* gene of *Bacillus licheniformis.*

15. The method of any of claims 1 – 13, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered encodes a D-alanine racemase and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID NO:12.

16. The method of any of claims 1 – 13, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more genes that are required for the host cell to grow on minimal medium supplemented with only one specific main carbon-source.

17. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is of a xylose operon, preferably the gene(s) is homologous to the *xylA* gene from *Bacillus subtilis*, and most preferably the gene(s) is homologous to one or more genes of the xylose isomerase operon of *Bacillus licheniformis.*

18. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered encodes a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably the gene(s) encodes an UDP-galactose epimerase (EC 5.1.2.3), more preferably the gene(s) is homologous to *galE* of a *Bacillus*, and most preferably the gene is *galE* of *Bacillus licheniformis.*

19. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more gene(s) of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both, more preferably the gene(s) is one or more genes homologous to the *gntK* or *gntP* genes from *Bacillus subtilis*, and most preferably the gene(s) is the *gntK* or *gntP* gene from *Bacillus licheniformis.*

20. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more gene(s) of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both and is at least 75%

identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to any of the *gntK* and *gntP* sequences of *Bacillus licheniformis*.

21. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more gene(s) of a glycerol operon, preferably the gene(s) encodes a glycerol uptake facilitator (permease), a glycerol kinase, or a glycerol dehydrogenase, more preferably the gene(s) is one or more genes homologous to the *glpP, glpF, glpK,* and *glpD* genes from *Bacillus subtilis*, and most preferably the gene(s) is one or more genes of *glpP, glpF, glpK,* and *glpD* genes from *Bacillus licheniformis* shown in SEQ ID NO:26.

22. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more gene(s) of a glycerol operon, preferably the gene(s) encodes a glycerol uptake facilitator (permease), a glycerol kinase, or a glycerol dehydrogenase, and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to any of the *glpP, glpF, glpK,* and *glpD* sequences of *Bacillus licheniformis* shown in SEQ ID NO:26.

23. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more gene(s) of an arabinose operon, preferably the gene(s) encodes an arabinose isomerase, more preferably the gene(s) is homologous to the *araA* gene from *Bacillus subtilis*, and most preferably the gene(s) is the *araA* gene from *Bacillus licheniformis* shown in SEQ ID NO:38.

24. The method of claim 16, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered is one or more gene(s) of an arabinose operon, preferably the gene(s) encodes an arabinose isomerase, and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *araA* sequence of *Bacillus licheniformis* shown in SEQ ID NO:38.

25. The method of any of claims 1 – 13, wherein the conditionally essential chromosomal gene(s) of the host cell encodes one or more polypeptide(s) involved in amino acid synthesis, and the non-functionality of the gene(s) renders the cell auxotrophic for one or more amino

acid(s), and wherein restoration of the functionality of the gene(s) renders the cell prototrophic for the amino acid(s).

26. The method of claim 25, wherein the conditionally essential chromosomal gene(s) of the host cell encodes one or more polypeptide(s) involved in lysine or methionine synthesis, more preferably the gene(s) is homologous to the *metC* or the *lysA* genes from *Bacillus subtilis*, and most preferably the gene(s) is the *metC* or the *lysA* gene from *Bacillus licheniformis.*

27. The method of claim 25, wherein the conditionally essential chromosomal gene(s) of the host cell is at least 75% identical, preferably 85% identical, more preferably 95% identical and most preferably at least 97% identical to the *metC* sequence of *Bacillus licheniformis* shown in SEQ ID NO:42 or the *lysA* sequence of *Bacillus licheniformis* shown in SEQ ID NO:48.

28. The method of any of claims 1 – 27, wherein the host cell is a Gram-positive bacterial cell, preferably a *Bacillus* cell, and most preferably a *Bacillus* cell chosen from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis.*

29. The method of any of claims 1 – 28, wherein the DNA construct is a plasmid.

30. A DNA construct comprising:

i) an altered non-functional copy of a conditionally essential chromosomal gene(s) from a host cell, preferably the copy is partially deleted; and

ii) at least one copy of a gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence located on the host cell chromosome adjacent to the conditionally essential gene(s) of i).

31. The DNA construct of claim 30, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered in i) encodes a D-alanine racemase, preferably the gene(s) is a *dal* homologue from a *Bacillus* cell, more preferably the gene is homologous to *dal* from *Bacillus subtilis*, and most preferably the gene is the *dal* gene of *Bacillus licheniformis.*

32. The DNA construct of claim 30, wherein the conditionally essential chromosomal gene(s) of the host cell that is altered in i) encodes a D-alanine racemase and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID NO:12.

33. The DNA construct of claim 30, wherein the altered non-functional copy of a conditionally essential chromosomal gene(s) from a host cell is one or more gene(s) that is required for the host cell to grow on minimal medium supplemented with only one specific main carbon-source.

34. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s) is one or more genes of a xylose operon, preferably the gene(s) is homologous to the *xylA* gene from *Bacillus subtilis*, and most preferably the gene(s) is homologous to one or more genes of the xylose isomerase operon of *Bacillus licheniformis*.

35. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s) encodes a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably the gene(s) encodes an UDP-galactose epimerase (EC 5.1.2.3), more preferably the gene(s) is homologous to the *galE* gene of *Bacillus subtilis*, and most preferably the gene(s) is the *galE* gene of *Bacillus licheniformis*.

36. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s) is one or more genes of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both, more preferably the gene(s) is homologous to the *gntK* or *gntP* genes from *Bacillus subtilis,* and most preferably the gene(s) is one or more genes of *gntK* and *gntP* from *Bacillus licheniformis.*

37. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s)is one or more gene(s) of a glycerol operon, preferably the gene(s) encodes a glycerol uptake facilitator (permease), a glycerol kinase, or a glycerol dehydrogenase, more preferably the gene(s) is one or more genes homologous to the *glpP, glpF, glpK,* and *glpD* genes from *Bacillus subtilis*, and most preferably the gene(s) is one or more genes of *glpP, glpF, glpK,* and *glpD* genes from *Bacillus licheniformis* shown in SEQ ID NO:26.

38. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s) is one or more gene(s) of a glycerol operon, preferably the gene(s) encodes a glycerol uptake facilitator (permease), a glycerol kinase, or a glycerol dehydrogenase, and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to any of the *glpP, glpF, glpK,* and *glpD* sequences of *Bacillus licheniformis* shown in SEQ ID NO:26.

39. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s) is one or more gene(s) of an arabinose operon, preferably the gene(s) encodes an arabinose isomerase, more preferably the gene(s) is homologous to the *araA* gene from *Bacillus subtilis,* and most preferably the gene(s) is the *araA* gene from *Bacillus licheniformis* shown in SEQ ID NO:38.

40. The DNA construct of claim 33, wherein the conditionally essential chromosomal gene(s) is one or more gene(s) of an arabinose operon, preferably the gene(s) encodes an arabinose isomerase, and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *araA* sequence of *Bacillus licheniformis* shown in SEQ ID NO:38.

41. The DNA construct of claim 30, wherein the conditionally essential chromosomal gene(s) encodes one or more polypeptide(s) involved in amino acid synthesis, and where and the non-functionality of the gene(s) when present in a cell with no other functional copy(ies) of the gene(s) renders the cell auxotrophic for one or more amino acid(s), and wherein restoration of the functionality of the gene(s) renders the cell prototrophic for the amino acid(s)

42. The DNA construct of claim 41, wherein the conditionally essential chromosomal gene(s) encodes one or more polypeptide(s) involved in lysine or methionine synthesis, more preferably the gene(s) is homologous to the *metC* or the *lysA* genes from *Bacillus subtilis,* and most preferably the gene(s) is the *metC* or the *lysA* gene from *Bacillus licheniformis.*

43. The DNA construct of claim 41, wherein the conditionally essential chromosomal gene(s) is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at

least 97% identical to the *metC* sequence of *Bacillus licheniformis* shown in SEQ ID NO:42 or the *lysA* sequence of *Bacillus licheniformis* shown in SEQ ID NO:48.

44. A host cell comprising at least two copies of a gene of interest stably integrated into the chromosome, where at least one copy is integrated adjacent to a conditionally essential *locus* and wherein the cell is obtainable by any of the methods defined in claims 1 – 29.

45. The cell of claim 44, wherein the gene of interest is separated from the conditionally essential *locus* by no more than 1000 basepairs, preferably no more than 750 basepairs, more preferably no more than 500 basepairs, even more preferably no more than 250 basepairs, and most preferably no more than 100 basepairs.

46. The cell of claims 44 or 45, which contains substantially no exogenous DNA, preferably less than 500 basepairs per integrated gene of interest, more preferably less than 300 bp, even more preferably less than 100 bp, still more preferably less than 50 bp, more preferably less than 25 bp per integrated gene of interest, or most preferably no exogenous DNA.

47. The cell of claims 44 or 45, which contains only endogenous DNA.

48. The cell of any of claims 44 - 47, which is a Gram-positive bacterial cell, preferably a *Bacillus* cell, and most preferably a *Bacillus* cell chosen from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*.

49. The cell of any of claims 44 – 48, wherein a copy of the gene of interest is integrated adjacent to a gene encoding a D-alanine racemase, preferably a gene homologous to the *dal* gene from *Bacillus subtilis*, more preferably a gene at least 75% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID NO:12, even more preferably a gene at least 85% identical, more preferably at least 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID NO:12.

50. The cell of any of claims 44 – 49, wherein a copy of the gene of interest is integrated adjacent to a gene that is required for the host cell to grow on minimal medium supplemented with only one specific main carbon-source.

51. The cell of claim 50, wherein a copy of the gene of interest is integrated adjacent to a gene of a xylose operon, preferably adjacent to genes homologous to the *xylR* or *xylA* genes from *Bacillus subtilis*, and most preferably adjacent to *xylR* or *xylA* from *Bacillus licheniformis.*

52. The cell of claim 50, wherein a copy of the gene of interest is integrated adjacent to a gene encoding a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably adjacent to a gene encoding an UDP-galactose epimerase (EC 5.1.2.3), more preferably adjacent to a gene homologous to the *galE* gene from *Bacillus subtilis*, and most preferably adjacent to *galE* from *Bacillus licheniformis.*

53. The cell of claim 50, wherein a copy of the gene of interest is integrated adjacent to a gene of a gluconate operon, preferably adjacent to a gene that encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease, more preferably adjacent to a gene homologous to a *Bacillus subtilis* gene chosen from the group consisting of *gntR, gntK, gntP,* and *gntZ*, and most preferably adjacent to *gntR, gntK, gntP,* or *gntZ* from *Bacillus licheniformis.*

54. The cell of claim 50, wherein a copy of the gene of interest is integrated adjacent to a gene of a glycerol operon, preferably the gene encodes a glycerol uptake facilitator (permease), a glycerol kinase, or a glycerol dehydrogenase, more preferably the gene is homologous to the *glpP, glpF, glpK,* or *glpD* gene from *Bacillus subtilis*, and most preferably the gene is the *glpP, glpF, glpK,* or *glpD* gene from *Bacillus licheniformis* shown in SEQ ID NO:26.

55. The cell of claim 50, wherein a copy of the gene of interest is integrated adjacent to a gene of an arabinose operon, preferably the gene encodes an arabinose isomerase, more preferably the gene is homologous to the *araA* gene from *Bacillus subtilis*, and most preferably the gene is the *araA* gene from *Bacillus licheniformis* shown in SEQ ID NO:38.

56. The cell of any of claims 44 – 50, wherein a copy of the gene of interest is integrated adjacent to a gene which encodes one or more polypeptide(s) involved in amino acid synthesis,

and the non-functionality of the gene(s) renders the cell auxotrophic for one or more amino acid(s), and wherein restoration of the functionality of the gene(s) renders the cell prototrophic for the amino acid(s).

57. The cell of claim 56, wherein a copy of the gene of interest is integrated adjacent to a gene which encodes one or more polypeptide(s) involved in lysine or methionine synthesis, more preferably the gene(s) is homologous to the *metC* or the *lysA* genes from *Bacillus subtilis*, and most preferably the gene(s) is the *metC* or the *lysA* gene from *Bacillus licheniformis.*

58. The cell of claim 56, wherein a copy of the gene of interest is integrated adjacent to a gene which is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *metC* sequence of *Bacillus licheniformis* shown in SEQ ID NO:42 or the *lysA* sequence of *Bacillus licheniformis* shown in SEQ ID NO:48.

59. The cell of any of claims 44 – 58, wherein the gene of interest encodes an enzyme, preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme, and more preferably an enzyme with an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase.

60. The cell of any of claims 44 – 58, wherein the gene of interest encodes an antimicrobial peptide, preferably an anti-fungal peptide or an anti-bacterial peptide.

61. The cell of any of claims 44 – 58, wherein the gene of interest encodes a peptide with biological activity in the human body, preferably a pharmaceutically active peptide, more preferably insulin/pro-insulin/pre-pro-insulin or variants thereof, growth hormone or variants thereof, or blood clotting factor VII or VIII or variants thereof.

62. The cell of any of claims 44 – 61, wherein no antibiotic markers are present.

63. A *Bacillus licheniformis* cell, wherein at least two conditionally essential genes are rendered non-functional, preferably the genes are chosen from the group consisting of *xylA*, *galE*, *gntK*, *gntP*, *glpP*, *glpF*, *glpK*, *glpD*, *araA*, *metC*, *lysA*, and *dal*.

64. Use of a cell as defined in claim 63 in a method as defined in any of claims 1 – 29.

65. A cell comprising a DNA construct as defined in claims 30 – 43.

66. A process for producing an enzyme of interest, comprising cultivating a cell as defined in any of claims 44 – 62 under conditions appropriate for producing the enzyme, and optionally purifying the enzyme.